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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : G01N 33/68, 33/543		A1	(11) International Publication Number: WO 00/29850 (43) International Publication Date: 25 May 2000 (25.05.00)
(21) International Application Number: PCT/FI99/00897		(81) Designated States: JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 27 October 1999 (27.10.99)		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 982481 17 November 1998 (17.11.98) FI			
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(54) Title: AN IMMUNOASSAY FOR THE DETERMINATION OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN BOVINE			
(57) Abstract			
<p>The present invention relates to an immunometric method for the determination of prion protein (PrP) in a body tissue or body fluid sample from a bovine animal, said method comprising the steps of subjecting the PrP to a treatment in order to facilitate its extraction and binding to antibodies; and subjecting the PrP from the foregoing step to a) a capture antibody bound or capable of binding to a solid phase, and b) a detecting antibody; and quantifying the signal from the detecting antibody bound to the solid phase. Furthermore, the invention concerns a method for diagnosing bovine spongiform encephalopathy (BSE) in a bovine animal.</p>			

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AN IMMUNOASSAY FOR THE DETERMINATION OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN BOVINE

FIELD OF THE INVENTION

5

This invention relates to an immunoassay for the determination of transmissible spongiform encephalopathies in bovine. More particularly, the invention concerns a novel method for the detection of the prion protein, a form of which is a diagnostic marker for the bovine spongiform encephalopathy (BSE).

10

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the 15 practice, are incorporated by reference.

The TSE family of diseases

Scrapie, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Sheinker (GSS) 20 syndrome and related diseases of mink (transmissible mink encephalopathy), mule deer and elk (chronic wasting disease) are classified as the transmissible degenerative (or spongiform) encephalopathies (TSE's). New species have been affected in recent years including cattle (bovine spongiform encephalopathy), cats (feline spongiform encephalopathy) and a variety of captive zoo felines and 25 antelope and a new form of CJD in man has recently emerged. Iatrogenic transmission of CJD in man occurs and these diseases can be transmitted from affected to healthy animals by inoculation or by feeding diseased tissues. The following text describing these diseases is taken from a review by Hope, 1998 (see reference 31).

30

Scrapie

Scrapie of sheep has been known in Europe for centuries and has spread to most parts of the world, excluding Australasia and Argentina, with the migrations of man and his livestock. It is characterised by altered behaviour, hypersensitivity to sound or touch, loss of condition, pruritus and associated fleece loss and skin abrasions and in-coordination of the hind limbs. Diagnosis is confirmed *post-mortem* by examination of brain tissue for a triad of histopathological signs - vacuolation, loss of neurones and gliosis¹.

10

Scrapie has been reported in most breeds of sheep and, within a flock, it appears to occur in related animals. The natural clinical disease has a median peak incidence in flock animals of 3.5 years, with a range of 2.5 to 4.5 years covering the vast majority of cases². For most of this period, the infected animal is clinically normal and indistinguishable from its un-infected flockmates. The within-flock incidence of clinical disease is usually 1-2 cases per 100 sheep per year but there have been several instances of 40-50% of animals of a flock succumbing to the disease within a year. A number of genetic markers have recently been identified as risk factors and the introduction of gene typing has greatly facilitated interpretation of field studies on the incidence of natural and experimental disease³.

Creutzfeldt-Jakob disease

Creutzfeldt-Jakob disease is a progressive dementia with clinical signs suggesting dysfunction of the cerebellum, basal ganglia and lower motor neurones. It is associated with gradual mental deterioration leading to dementia and confusion, and a progressive impairment of motor function. Most patients die within six months of onset of clinical signs and there are no verified cases of recovery. Pathologically the lesions of the brain included variable vacuolation of the neuropil, astrocytosis

and, in about 10% of CJD cases, amyloid plaques. Gerstmann-Straussler syndrome is a familial variant of CJD with an extended, clinical time course.

The incidence of CJD-related disease in man is remarkably constant at 0.5-1 cases per million of population per year throughout the world and so is not linked to the incidence of any of the animal diseases. This low incidence casts doubt on the role of infection in its propagation within the population (but see below). About one in seven of cases are familial and linked to mutations in the open reading frame of the PrP (prion protein) gene. There has been a large amount of clinical and pathological studies on human cases of neurological disease which seem to be associated with these rare mutations of the PrP gene (for a review, see⁴). In some families, there is complete penetrance of the phenotype and so the mutation is regarded as the cause of the disease. Apart from iatrogenic cases induced by transplantation of infected tissues or inoculation of contaminated pharmaceuticals of human origin, there is no epidemiological evidence for horizontal transmission of the disease. A stochastic event involving conversion of the PrP protein to its disease-associated isoform or the chance mutation of a benign, ubiquitous viral-like agent are two mechanisms which have been suggested to explain the incidence of sporadic cases (see section on the nature of the agent). There is no cure for the clinical condition although genetic counselling, where applicable, may effectively prevent transmission of disease from one generation to the next.

There is considerable clinical and pathological heterogeneity in the human prion diseases and although genetic typing and nucleotide sequencing of the PrP ORF (ORF = Open reading Frame) has provided some unifying concepts, mutation in the PrP protein does not appear to be the whole story. Other genetic factors including linkage to the E4 allele of ApoE gene have been implicated as risk factors for the occurrence of CJD.

Emerging human TSE's

Fatal familial insomnia (FFI) has emerged as the newest member of the human transmissible encephalopathies. It is characterised by a dysfunction of the autonomic nervous system usually presenting with insomnia and problems of appetite, temperature and blood pressure regulation. At post-mortem, the pathology of the brain is mostly neuronal loss and degeneration of the thalamus with little or no vacuolation of the neuropil. Its classification as a prion disease was originally based on its association with an asparagine (N) to aspartic acid (D) mutation at codon 178 of the PrP gene, a mutation which is also linked to a classical form of CJD. Which of the two phenotypes prevails appears to depend on the amino-acid encoded by codon 129 of the same PrP allele : in FFI, codon 129 encodes methionine while in CJD, codon 129 encodes valine. Homozygosity at codon 129 also appears to be risk factor in the development of sporadic CJD, but each polymorphism at this codon is fairly common and not thought to be pathogenic per se. The classification of FFI has been confirmed by transmission of disease to laboratory mice⁵.

In March 1996, the UK government announced their concern about a new variant of CJD and details of these cases have subsequently appeared⁶. To date, twenty-eight cases of CJD have been identified in the UK with a new neuropathological and clinical profile - extensive PrP deposition, cerebellar amyloid plaques, spongiform change most evident in the basal ganglia and thalamus, prolonged duration (up to 2 years), atypical EEG, early ataxia, behavioural and psychiatric disturbances. Similar cases have not been identified in archival patient files or elsewhere in Europe (apart from a single French case⁷) and there would appear to be a risk factor for this variant unique to the UK. Its co-incidence with a novel bovine TSE (BSE) which is largely restricted to the UK has led to speculation, as yet unproven, that this new form of CJD represents a cross-species transmission of infection from cattle.

Bovine spongiform encephalopathy

Bovine spongiform encephalopathy (BSE) has devastated the UK cattle industry for the past decade⁸. From isolated cases first reported in 1986 and some

5 retrospectively identified in May 1985, a major epidemic was underway by 1988 which has to date claimed over 180 000 cattle within the British Isles. Some other countries have also confirmed cases : Switzerland (450+), Ireland (700+), Portugal (300+), France and Germany with one or two cases in Italy, Denmark, Canada, the Netherlands, Oman and the Falkland Islands.

10

The disease produces a progressive degeneration of the central nervous system and was named because of the sponge-like appearance of BSE-brain tissue when seen under the light microscope⁹. Warning signs of the illness include changes in the behaviour and temperament of the cattle. The affected animal becomes increasingly

15 apprehensive and has problems of movement and posture, especially of its hindlimbs. The cow (or bull) has increased sensitivity to touch and sound, loss of weight and, as the disease takes hold of its nervous system, a creeping paralysis sets in. This clinical phase of BSE lasts from a fortnight to over six months. Although the majority of animals affected have been dairy cows, this neurological disease can

20 occur in either sex with a modal age of onset of 4-4.5 years (range 1.8 - 18 years).

Most cases of BSE have occurred in cattle between the ages of 3 and 5 years and for most of its development time the disease gives no tell-tale sign of its presence¹⁰.

25 The neurological lesions in BSE-affected cow brains are virtually identical to those found in scrapie-affected sheep and include the spongiform change which gives BSE its name. From its clinical and neuropathological signs, BSE was immediately suspected to belong to the scrapie family of transmissible spongiform encephalopathies. This has been confirmed by biochemical studies¹¹ and by experimental transmission of BSE to mice¹², sheep and goats¹³ amongst other

30 species.

The prion protein (PrP)

5 The conversion of a normal membrane glycoprotein, the cellular prion protein or PrP^C , to an aggregated, insoluble isoform, PrP^{Sc} , is a key process in the pathogenesis of BSE, scrapie and other transmissible spongiform encephalopathies (TSE's). The specific detection of PrP^{Sc} forms the basis for biochemical diagnosis of these diseases. Folding differences in the abnormal isoform of the prion protein (PrP^{Sc})

10 can be investigated by probing the conformation of the protein in diseased tissues by proteolysis under conditions where the normal protein is either destroyed and drastically reduced in amount prior to detection by SDS-PAGE/immunoblotting or ELISA techniques.

15 *The primary structure of PrP*

The amino acid sequence of mouse, human and bovine PrP are given below using the IUPAC single letter code for amino-acids :

20 Mouse (Laboratory strain, NZW) (SEQ NO: 1):

(Amino-)KKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGTWGQPH
GGGWGQPHGG SWGQPHGGSW GQPHGGGWGQ GGGTHNQWNK
PSKPKTNLKH VAGAAAAGAV VGGLGYYMLG SAMSRPIMHF

25 GNDWEDRYYR ENMYRYPNQV YYRPVDQYSN QNNFVHDCVN
ITIKQHTVTT TTKGENFTET DVKMMERVVE QMCVTQYQKE
SQAYYDGRRS(-GPI)

Cattle (*Bos bovis*) (SEQ NO: 2):

(Amino-)KKRPKP GGGWNTGGSR YPGQGSPGGN RYPPQGGGGW
GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GWGQPHGGGG

5 WGQGGTHGQW NKPSKPKNM KHVAGAAAAG AVVGLGGYM
LGSAMSRPLI HFGSDYEDRY YRENMRYPN QVYYRPVDQY
SNQNNFVHDC VNITVKEHTV TTTKGENFT ETDIKMMERV
VEQMCITQYQ RESQAYYQRG A(-GPI)

10 Human (SEQ NO: 3):

(Amino-)KKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQPH
HGGGWGQPHG GGWGQPHGGG WGQPHGGGWG QGGGTHSQWN
KPSKPKNMK HMAGAAAAGA VVGLGGYML GSAMSRPIIH

15 FGSDYEDRYY RENMRYPNQ VYYRPMDEYS NQNNFVHDCV
NITIKQHTVT TTTKGENFTE TDVKMMERVV EQMCITQYER
ESQA YYQRGS(-GPI)

Notable structural features which are found in all mammalian prion proteins are a
20 series of eight amino-acid repetitive sequences (*PH GGGWGQ*) (SEQ NO: 4), a
cystine bridge (-C....C-), two sites for N-glycan attachment (..N..) and a C-terminal
phospho-inositol glycolipid membrane anchor (-GPI). Each species may have
several alleles of the gene encoding PrP, some of which are linked to the incidence
of disease or the survival time of individuals exposed to infection.

25

The prion protein is expressed in many different cells but is found in greatest
abundance associated with the neurones of the central nervous system.
Consequently, the abnormal form of PrP accumulates predominantly in the brain
although it can also be detected in extra-neuronal tissues such as the tonsil and spleen

early in the development of disease. It is therefore correct to say that the total concentration of PrP is greater in affected tissues compared to healthy tissues.

In cell culture, the PrP protein is cycled to and from the cell surface via the
5 endosome-lysosome system; during this process the protein appears to undergo proteolytic cleavage between residues 109 and 112. To what extent this cleavage occurs *in vivo* is unknown although C-terminal fragments of PrP similar to those expected to result from the lysis of this peptide bond have been seen in deposits of mouse and human PrP^{Sc}. The exact site of cleavage may be related to the phenotype
10 of disease or strain of infectious agent; this is an area of current investigation.

Previous work on detection of PrP

Ever since the prion protein (PrP) was discovered, measurements of prions and PrP
15 have been made relating the number of protein molecules per infectious particle. This is usually quoted as 100 000 PrP molecules per infectious particle if measured by intracerebral inoculation in an homologous rodent bioassay; for example, using the 263K strain of agent in hamsters or the ME7 strain in mice, etc. The detection of PrP^{Sc} as fibrils^{14, 15} or by immunochemical methods¹⁶⁻²² can be used as a diagnostic
20 test for TSE's either at post-mortems or in the live animal. A preliminary validation of this approach using fibril or PrP ICC (ICC = immunocytochemistry) to monitor the oral pathogenesis of BSE in cattle has recently been published²³. Western or dot blotting can currently detect 10-100 pg (10⁸-10⁹ molecules) of PrP^{Sc} and these methods are about the same sensitivity as an heterologous bioassay of bovine or
25 human tissue for BSE infectivity in mice. For example, typically, 1 gram of BSE-affected brain contains 1 microgram of PrP^{Sc}¹¹ and 10^{3.5} LD₅₀ units/g of infectivity²⁴. This is equivalent to a specific infectivity of 1 infectious particle/10⁹ molecules. Higher specific infectivities may be found in transmissions between the same species^{25, 26} but from these calculations it is clear mouse bioassays offer no
30 greater sensitivity than PrP immunochemical assays for detection of BSE infectivity.

Improving the sensitivity of the assay system may shed light on the scientific conundrum of why lateral or maternal transmission of BSE occurs²⁶ in the (apparent) absence of infectivity (and PrP^{Sc}) in milk²⁷, blood, placenta and other peripheral tissues of the BSE-infected cow²⁸.

5

The specificity of the FH11 and 3F4 monoclonal antibodies

The epitope specificities of FH11 and 3F4 are mapped out below (Birkett et al., unpublished; ²⁹). The use of FH11 for the immunocytochemical detection of PrP in 10 sheep brain tissue sections has been reported ³⁰; this report included its N-terminal specificity and the use of trypsin to enhance the detection of PrP^{Sc}.

FH11 (also BG4)

15 FH11, primary epitope

bov49/hum46 gnrypPQGGGgwg (SEQ NO: 5)

FH11 suspected secondary epitope

20

hum90	gqgggthsqwnk	(SEQ NO: 6)
ham90/mou89 /rat	gqgggthnqwnk	(SEQ NO: 7)
ovi94	gqggshsqwnk	(SEQ NO: 8)
bov94	gqggthgqwnk	(SEQ NO: 9)

25

3F4

ham103/hum103	skpktnMkhMagaaa	(SEQ NO: 10)
bov106/ovi106	skpktnMkhVagaaa	(SEQ NO: 11)

30

The seemingly essential part of the epitopes has been capitalised. The 'core' of the epitopes has been italicised.

SUMMARY OF THE INVENTION

5

According to one aspect, this invention concerns an immunometric method for the determination of prion protein (PrP) in a body tissue or body fluid sample from a bovine animal. According to the invention, the method comprises the steps of

- subjecting the PrP to a treatment in order to facilitate its extraction and binding to antibodies, and
- subjecting the PrP from the foregoing step to
 - a) a capture antibody bound or capable of binding to a solid phase, and
 - b) a detecting antibody, and
- quantifying the signal from the detecting antibody bound to the solid phase.

15

According to another aspect, this invention concerns a method for diagnosing bovine spongiform encephalopathy (BSE) in a bovine animal, said method comprising the determination of prion protein (PrP) in a body tissue or body fluid sample from said bovine animal, by the novel immunometric method of this invention. The presence of PrP is used as indication of BSE in said bovine animal.

According to still another aspect, this invention concerns a method for the *post-mortem* control of bovine bodies after slaughter in order to identify bodies showing BSE infection, said method comprising the determination of prion protein (PrP) in a body tissue or body fluid sample from said bovine body, by the novel immunometric method of this invention. The presence of PrP is used as indication of BSE in said bovine body.

30

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a calibration curve for hamster PrP.

5 Figure 2 shows a calibration curve for bovine PrP.

Figure 3 demonstrates an assay of PrP in hamster brain tissue where detected PrP concentration is plotted versus Proteinase K concentration in the experiment (filled bars: scrapie, and striped bars: normal).

10

Figure 4 demonstrates an assay of PrP in bovine brain tissue where detected PrP concentration is plotted versus Proteinase K concentration in the experiment (filled bars: BSE, and striped bars: normal).

15 Figure 5 demonstrates an assay of PrP in ovine brain tissue where detected PrP concentration is plotted versus Proteinase K concentration in the experiment (filled bars: scrapie, and striped bars: normal).

DETAILED DESCRIPTION OF THE INVENTION

20

This invention relates to a novel two-site immunometric assay for the measurement of prion protein (PrP). The method involves the use of a capture antibody that recognises an epitope, preferably an epitope in the N-terminal region of the molecule. The detecting antibody recognises preferably an epitope in the protease 25 resistant core of the PrP molecule that is occluded when the PrP is in an aggregated state. The use of a limited concentration of proteolytic enzyme facilitates the disaggregation of the PrP. This leads to an increase in the binding of the detector antibody.

The "N-terminal region" shall particularly mean the region defined by aa 50 to 95 of the PrP molecule.

It shall, however, be noted that either or both of the capture and detecting antibodies 5 may be directed to epitopes in the N-terminal region, the protease resistant core, or the C-terminal region of the PrP molecule.

The treatment of the PrP may include, for example, any of the following: (i) limited 10 proteolysis; (ii) addition of detergents; (iii) addition of chaotropic agents and/or solvents; (iv) variation of temperature; and (v) sonication. These treatments can be used individually or in combination.

The "protease resistant core of the PrP molecule" is the part of the PrP molecule 15 that is not degraded by proteolysis. The normal metabolic cleavage of the molecule is in the amino acid region 109 - 112. The cleavage caused by, for example, a proteolytic enzyme depends on the tissue, cell type, mammal etc.. For Proteinase K, the cleavage takes place in the region of position 90 for 263K strain in LVG hamsters.

20 The capture antibody as well as the detecting antibody are preferably both monoclonal antibodies.

The detecting antibody can be labelled with any detectable label. However, 25 according to a preferred embodiment, the label is a lanthanide chelate, wherein the detection is based on time-resolved fluorescence.

The sample can be, for example, brain tissue, spinal cord, lymphoid tissue, spleen, tonsil, whole blood or a blood fraction. For *post-mortem analyses*, brain tissue is a particularly preferred sample. For investigation of living bovine individuals, less

invasive tissue samples or body fluid samples must be used. As examples of body fluid samples can be mentioned whole blood or fractions thereof.

The use of the antibody FH11 as a capture reagent is new and nonobvious in any

5 assay system for the detection of PrP^{Sc} using ProteinaseK (PK). Conventional assay systems (e.g. Western Blot analysis) utilise sufficient PK to digest PrP down to its protease resistant core. This protease-resistant core does not possess the primary epitope for FH11 or any other antibody in the N-terminal region of the molecule.

10 The 3F4 antibody has been widely used to detect PrP in hamster and human tissue samples. However, it has never been shown to cross-react with ovine or bovine PrP in Western Blot analysis. Consequently, its use in an assay for the measurement of PrP in ovine or bovine tissue is new and nonobvious. It has proved to be a very useful reagent because of the increased sensitivity obtainable in DELFIA which

15 allows for the exploitation of the low cross-reaction of 3F4 for the ovine and bovine epitope.

The other novel aspect of the use of this antibody is the finding that the 3F4 epitope is in the 'core' region of the molecule which is involved in the aggregation process.

20 When PrP is in an aggregated state, the 3F4 epitope is occluded and is not available for antibody binding. When PrP is disaggregated by limited PK digestion, treatment with a chaotropic agent (e.g. > 2M guanidinium chloride) and/or detergent addition, the 3F4 epitope becomes exposed, the antibody binds and the signal increases.

25 PK at low dose may facilitate the measurement of highly aggregated PrP by:

1. Solubilisation of PrP which is achieved by the proteolytic cleavage of PrP from its GPI anchor which attaches the molecule to the cell membrane.
2. Limited proteolysis and removal of proteins associated with the aggregated PrP. These proteins include PrP itself and non-PrP binding proteins which have yet to be

30 identified.

3. The removal of binding proteins facilitates the disaggregation of PrP ideally but not necessarily in the presence of detergent with the concomitant exposure of epitopes within the core region of the molecule.

5 The PK effect may be achieved by the addition of limiting concentrations of PK. Increased concentrations of the enzyme will result in the cleavage of PrP resulting in the ultimate loss of the primary FH11 epitope.

10 The results of the immunometric method according to this invention can be used for different purposes. They can be used for diagnosing a bovine spongiform encephalopathy (BSE) in the bovine animal, which can be a living individual or a deceased individual.

15 An especially important field of use can be mentioned routine control of bovine bodies in slaughterhouses. A sample can be derived from each slaughtered body and analysed. Bodies showing BSE infection will be destroyed. This would be an effective method for preventing infected meat and other bovine-based products from being commercialised. On the other hand, unnecessary destruction of healthy bodies can be avoided. Therefore, this control method is of great economical value.

20

The invention is described in more detail by the following, non-limiting experiments.

EXPERIMENTAL SECTION

25

Materials and methods

1. DELFIA (trademark) reagents were obtained from: EG & G Wallac Ltd, 20 Vincent Avenue, Crownhill Business Centre, Crownhill, Milton Keynes MK8 OAB

30

1.1 Assay Buffer**Catalogue No. 1244-106**

Ready for use Tris-HCl buffered (pH 7.8) salt solution with bovine serum albumin,

5 bovine globulin, Tween 40, an inert red dye, and < 0.8 % sodium azide as preservative. Stored between 2-8 degrees until expiry date stated on vial label.

1.2 Wash Concentrate**Catalogue No. B117-100**

10

A 25-fold concentration of Tris-HCl buffered (pH 7.8) salt solution with Tween 20 and Germall II as preservative. Stored between 2-8 degrees Celsius until expiry date. Wash solution was prepared by diluting wash concentrate 25-fold (i.e. 40 mL concentrate diluted to 1 litre) with distilled water.

15

1.3 Enhancement Solution**Catalogue No. 1244-105**Ready for use with Triton X-100, acetic acid and chelators. Stored between 2-25
20 degrees Celsius until expiry date. Direct sunlight avoided.**1.4 Europium-labelling Reagent Kit****Catalogue No. 1244-302**

25 Each kit contains 0.2 mg labelling reagent plus Eu-standard, enhancement solution, stabiliser (purified BSA) for increasing the stability of the labelled protein, an uncoated microtitration strip plate, assay buffer and wash concentrate. The kit contents were sufficient for a labelling of up to 1 mg of protein. Stored between 2-8 degrees Celsius until expiry date.

30

1.5 Low Fluorescence Background uncoated Microtitre Plates
Catalogue No. 1244-550

5 DELFIA plates were in strip format 8 x 12 and manufactured by NUNC from a
plastic with a low fluorescent background. They had a high immunoglobulin
binding surface (MAXISORB).

2. Other reagents

10 2.1 ImmunoPure (A) IgG Purification Kit

Available from: Pierce and Warriner (UK) Ltd., 44 Upper Northgate Street, Chester,
Cheshire CH1 4EF Catalogue No. 44667

15 Contents:

1. ImmunoPure IgG binding buffer (1000 mL; pH 8.0) prepared from the
highest quality buffer salts, using ultra pure reagent grade water
and filtered (0.2 micron). The buffer contains EDTA as a
20 preservative.

2. ImmunoPure IgG elution buffer (500 ml; pH 2.8). The buffer has
been purged with nitrogen to exclude oxygen. The absence of oxygen
and low pH allowed good reagent stability without the use of a
25 preservative.

3. Protein A AffinityPak Columns, 5 x 1 ml columns.

2.2 Amicon Microconcentrators (Microcon 30)

Microcon concentrators employed Amicon's low binding, anisotropic, hydrophilic YM membrane. The Microcon-30 used a membrane with a molecular weight cut-off of 30,000 KDa. The devices were usable in any Eppendorf centrifuge (e.g. MSE Micro-Centaur Microfuge) and offer a simple, efficient, means of concentrating, 5 desalting and purifying proteins before and after labelling with lanthanide chelate (or biotin).

The low absorption characteristics of the YM membrane, and the device's component parts, together with an inverted recovery spin, combined to yield 10 unusually high recovery rates - typically 95% of the sample, with concentration factors as high as 100x.

Available from: Millipore UK Ltd, The Boulevard, Blackmore Lane, Watford WD1 8YW Catalogue No. 42409

15

2.3 Labelling Buffer

Sodium bicarbonate buffered saline (50 mmol/L) was prepared by dissolving 4.2 g 20 NaHCO₃ (Sigma S-8875) and 9.0 g NaCl (Sigma S-9625) in 1 litre of ultra pure water (Milli-Q or equivalent). The pH of the resulting solution is adjusted with NaOH (2N; Aldrich 22,146-5) to between 8.5 and 9.0. The solution was stored between 2-8 degrees Celsius.

2.4 Coating Buffer

25

Phosphate Buffered Saline (Sigma 1000-3) prepared by dissolving sachet contents in 1 litre of distilled water producing a solution containing NaCl (120 mmol/L), KCl (2.7 mmol/L) and phosphate buffer (10 mmol/L), pH 7.4 at 25 degrees Celsius. To 30 this solution sodium azide (Sigma S-2002; 0.1%) may be added. The solution was stored between 2-8 degrees Celsius.

2.5 Blocking Buffer

5 Phosphate Buffered Saline (Sigma 1000-3) with addition of sodium azide (Sigma S-2002; 0.1%) and bovine serum albumin (Sigma A-7888; 2%). Stored between 2-8 degrees Celsius.

3. Materials supplied by Institute of Animal Health

10 3.1 Monoclonal Antibodies

At the date of the invention, the most useful monoclonal antibodies available were:

(i) Capture Antibody - FH11

15 Approximate concentration: 3.9 mg/mL (purified IgG)

(ii) Detecting antibody- 3F4

Approximate concentration: 1 mg/mL (purified IgG)

20 3.2 Recombinant PrP Standard

3.2.1. GST-Hamster PrP Fusion Protein

25 The stock solution was estimated at 0.48 mg/mL and was stored in chaotropic buffer containing 4M Guanidinium hydrochloride at - 20 degrees Celsius.

Preparation of standards

Stock PrP-GST fusion protein was serially diluted ten-fold in assay buffer to give a series of standards as follows: (i) buffer blank; (ii) 0.48 ng/mL; (iii) 4.8 ng/mL; (iv) 48 ng/mL; (v) 480 ng/mL; and, (vi) 4800 ng/mL.

5 The standards were prepared fresh prior to each experiment.

3.2.2 Histidine-tagged Hamster PrP protein

Subsequent recombinant Hamster PrP protein comprised extracts of a

10 histidine-tagged PrP protein in chaotropic buffer solution. The protein concentration of these extracts ranged from 0.78 to 8 mg/mL. The stock solutions are stored at -20 degrees Celsius.

3.2.3 Recombinant Bovine PrP

15

Recombinant bovine PrP (4 mg/mL) stock solution was stored at -20 degrees Celsius. The stock solution was serially diluted in assay buffer to give a series of standards as follows: (i) buffer blank; (ii) 4 ng/mL; (iii) 40 ng/mL; (iv) 400 ng/mL; (v) 4000 ng/mL; and, (vi) 40,000 ng/mL. The standards were prepared fresh prior to 20 each experiment.

Purification of antibodies using immunopure (A) IgG kit

Approximately, 0.5 mL aliquots of: (i) mouse ascites (3F4); or (ii) culture

25 supernatant (FH11) were diluted with 1 mL of ImmunoPure IgG Binding Buffer and applied to an AffinityPak prepacked 1 mL column of Immobilized Protein A which had been pre-equilibrated with binding buffer. Chromatography was performed at room temperature and eluates were monitored at 280 nm using a UVICORD flow detection monitor connected to a flat bed recorder.

When all protein had been washed from the AffinityPak column, the specific IgG was eluted, following application of elution buffer. The presence of protein in the eluate was detected by the monitor and the buffer collected in a glass vial. The concentration of the eluted protein was estimated by the measurement of absorbance 5 at 280 nM using a spectrophotometer.

The column was regenerated by washing with 4 column volumes of 0.1 M citric acid, adjusted to pH 3.0 with 6 N NaOH. For storage, the column is washed with an additional 5 ml of water containing 0.02 % sodium azide.

10

Labelling selected antibodies with europium

Buffer exchange

15 Five hundred (500) μ L aliquots of the 3F4 monoclonal detecting antibody purified on Protein A were added to Microcon-30 sample reservoirs inserted into their Eppendorf vial holders. The tubes were capped and spun at 6000 rpm for 15 minutes. Subsequently, the eluates were removed to waste and further 400 μ L aliquots of labelling buffer added to the sample reservoirs. The tube were spun as 20 previously and the procedure repeated twice more after further additions of 400 μ L of labelling buffer.

25 After the final preparative centrifugation, the vials were separated from the sample reservoirs which were placed upside down in new vials. The devices were capped and spun by pulsing briefly to transfer the concentrate to the vial. The concentrated solutions of purified 3F4 IgG were diluted with labelling buffer to give an approximate concentration of 2-3 mg/mL IgG.

Labelling procedure

Approximately 1 mg of the concentrated purified specific 3F4 IgG preparation was added to a vial containing freeze-dried labelling reagent (0.2 mg). The vial was stoppered, the contents gently mixed by swirling and left at room temperature 5 overnight.

Purification

Separation of the europium-labelled 3F4 monoclonal antibody from unreacted 10 labelling reagent was effected using Microcon-30 micro-concentrators using a similar procedure to that described above. The buffer used in this purification, however, was Tris buffered saline (TBS; 50 nmol/L Tris-HCl buffer, pH 7.75, containing NaCl (0.9 %) and NaN₃ (< 0.1 %)). The purified europium-labelled antibody was stored in a glass vial.

15 For a more sophisticated separation, the bound label can be removed by gel filtration on a Sephadex G-50 column. Elution can be carried out with TBS (as above) and the column decontaminated by washing with phthalate buffer, 10 mmol/L, pH 4, containing 0.001 % DTPA. Possible aggregates present in the 20 protein fraction or formed during labelling sometimes cause elevated backgrounds in solid phase assays. These aggregates can be removed by using suitable gel filtration media (e.g. Sepharose 6B or Sephacryl S400).

Characterization of the labelled protein

25 The europium content of the labelled antibody was determined by diluting the labelled product in enhancement solution (1:10,000 v/v) and measuring the signals obtained in the time-resolved fluorometer. The counts are compared with the signal generated from a 1:100 dilution of europium standard in enhancement solution 30 (equivalent to 1 nmol/L Eu³⁺).

The protein concentrations of the labelled antibody was determined using a spectrophotometer at 280 nm. This concentration must be adjusted by correcting for the absorbance generated by the thiourea bond (0.008A/1 μ mol/L of Eu at levels of 5 less than 20 Eu/IgG).

Storage

For good stability the labelled antibodies were stored at high concentration and in 10 the absence of competing metals (or chelators) in the buffer. A concentrated solution (0.050 mg/mL) was stored at +4 degrees Celsius. The stability can be increased by adding small amounts (about 0.1%) of stabiliser contained in the labelling kit which consists of BSA which has been purified free from heavy metal contaminants.

15

The measurement of prion protein using purified monoclonal FH11 as capture antibody and europium-labelled 3F4 as detector

Coating the capture antibody

20

On the day prior to the DELFIA assay, the required number of microtitration plates were coated with purified monoclonal FH11 diluted in coating buffer (1:1000 v/v). Two hundred (200) μ L of diluted antibody solution were added to each well using a multichannel pipette. The plates were covered and incubated overnight at 4 degrees 25 Celsius. Next day, the plates were washed three times using the WALLAC plate washer.

Blocking the plates

Two hundred (200) μ L blocking buffer were added to each well using the multichannel pipette. The plates were incubated for 1 hour at room temperature on the plate shaker.

5 **Addition of standard or sample**

Two hundred (200) μ L of diluted recombinant standard or tissue extract were pipetted into the appropriate microtitre wells. The plates incubated for 1 hour at room temperature on the plate shaker. Subsequently, the plates were washed three 10 times using the plate washer and tapped dry on absorbant paper.

Addition of europium-labelled detecting antibody

Two hundred (200) μ L of europium-labelled 3F4 monoclonal detecting antibody 15 diluted in assay buffer (1:5000 v/v) were added to each well using an Eppendorf repeating pipette. The plates were incubated for 1 hour at room temperature on the plate shaker. The plates were washed six times and tapped dry on absorbant paper.

Addition of enhancement solution

20

Two hundred (200) μ L of enhancement solution were added to each well using the designated Socorex dispenser. The plates were incubated for 5 minutes on the plate shaker at room temperature and transferred to the 1234 Plate Fluorometer.

25 **Calibration Curves**

Hamster calibration curves are shown in Figure 1. Bovine calibration curves are shown in Figure 2.

Brain tissue extraction protocol**Tissue homogenisation**

5 Brain tissue was carefully weighed and transferred to sterile Dounce homogeniser. 1.5 mL of homogenisation buffer was added to each gram of brain tissue.

Homogenisation buffer A: PBS (without detergent) or B: 1MGdnCL in 50mM Tris-HCl pH 7.5 containing 0.5% sulphabetaine (3-14)

10

The brain tissue was homogenised with 10 - 15 passes using the 'loose' pestle.

15 1.5 mL of homogenisation buffer was added and the brain tissue was homogenised for another 10 - 15 passes using the 'tight' pestle. The resulting mixture was a creamy emulsion and corresponds to a 25% brain homogenate based on the assumption that 1 gram of brain tissue is equivalent to 1 mL.

An aliquot of the emulsion was transferred to polycarbonate ultra-centrifuge tubes (Beckman).

20

Proteolytic digestion

25 Proteinase K (Sigma or Boehringer) was carefully weighed and dissolved in PBS to provide an appropriate stock solution. An aliquot of the PK solution was added to the emulsion in the polycarbonate tubes to give a final concentration of PK within the range 0 to 100 µg/mL. The tubes were sealed with PVC tape and incubated at 37°C for 30 minutes with gentle agitation. The digestion was stopped by the addition of 10 µL Pefabloc SC (0.5 M solution in PBS).

First ultracentrifugation

The polycarbonate tubes were transferred to a Beckman TL100.3 rotor and centrifuged @ 100,000 rpm for 8 minutes at 22°C in TL100 ultracentrifuge.

5 The supernatant was removed and retained (primary supernatant). Extraction buffer was added equal to the volume of supernatant removed. Extraction buffer: 6MGdnHCl in 50mM Tris-HCl pH 7.5 containing 0.5% sulphabetaine (3-14) The pellet was resuspended by trituration with a plastic disposable pasteur pipette. The samples were incubated for 5 minutes at room temperature.

10

Second ultracentrifugation

The polycarbonate tubes were centrifuged @ 100,000 rpm for a further 8 minutes at 22°C in TL100 ultracentrifuge. The supernatant is designated the secondary extract.

15

Results

The time-resolved fluorescence immunoassay of PrP in brain tissue extracts from scrapie-infected and normal hamsters is shown in Figure 3., the time-resolved fluorescence immunoassay of PrP in brain tissue extracts from BSE-infected and normal cattle is shown in Figure 4, and the time-resolved fluorescence immunoassay of PrP in brain tissue extracts from scrapie-infected and normal sheep is shown in Figure 5.

25 A typical PK titration experiment involved the production 10 mL of total brain homogenate. This was equivalent to 2.5 grams of brain tissue (approximately 2.5 brains). 10 aliquots (0.8 mL) were transferred to separate polycarbonate tubes and added the same volume of PK at 10 different concentrations to ensure that each tube was comparable. This full experiment was repeated 4 times with normal and scrapie 30 hamster brains and gave essentially identical results both in terms of concentration

and PK titration profile. This approach was adopted for bovine and ovine brain tissue and similar results were obtained.

In this extraction procedure, PK has a dual effect. It not only digests PrPc but

5 facilitates the measurement of PrP in the primary and secondary extracts.

For example, 5 μ g/mL of PK 'releases' up to 100 times more PrP in hamster brain tissue than can be measured in samples processed without PK or in the presence of 100 μ g/mL PK.

10

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are

15 illustrative and should not be construed as restrictive.

REFERENCES

1. Hadlow, W.J. Neuropathology and the scrapie-kuru connection. *Brain Pathology* 5, 27-31 (1995).
- 5 2. Hunter, N., Foster, J.D. & Hope, J. Natural scrapie in British sheep breeds, ages and PrP gene polymorphisms. *Vet. Rec.* 130, 389-392 (1992).
- 10 3. Goldmann, W., Hunter, N., Smith, G., Foster, J. & Hope, J. PrP genotype and agent effects in scrapie - change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. *J. Gen. Virol.* 75, 989-995 (1994).
- 15 4. Brown, P., et al. Human spongiform encephalopathy - the national-institutes-of-health series of 300 cases of experimentally transmitted disease. *Annals of Neurology* 35, 513-529 (1994).
5. Tateishi, J., et al. First experimental transmission of fatal familial insomnia. *Nature* 376, 434-435 (1995).
- 20 6. Will, R.G., et al. A New Variant Of Creutzfeldt-Jakob-Disease In the Uk. *Lancet* 347, 921-925 (1996).
- 25 7. Chazot, G., et al. New Variant Of Creutzfeldt-Jakob-Disease In a 26-Year-Old French Man. *Lancet* 347, 1181 (1996).
8. Bradley, R. & Wilesmith, J.W. Epidemiology and control of bovine spongiform encephalopathy (bse). *British Medical Bulletin* 49, 932-959 (1993).
- 30

9. Wells, G.A.H., *et al.* A novel progressive spongiform encephalopathy in cattle. *Vet. Rec.* **121**, 419-420 (1987).

5 10. Wilesmith, J.W., Wells, G.A.H., Cranwell, M.P. & Ryan, J.B.M. Bovine spongiform encephalopathy - epidemiological-studies. *Vet. Rec.* **123**, 638-644 (1988).

11. Hope, J., *et al.* Fibrils from brains of cows with new cattle disease contain scrapie-associated protein. *Nature* **336**, 390-392 (1988).

10

12. Fraser, H., McConnell, I., Wells, G.A.H. & Dawson, M. Transmission of bovine spongiform encephalopathy to mice. *Vet. Rec.* **123**, 472 (1988).

15

13. Foster, J.D., Hope, J. & Fraser, H. Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet. Rec.* **133**, 339-341 (1993).

14. Gibson, P.H., Somerville, R.A., Fraser, H., Foster, J.D. & Kimberlin, R.H. Scrapie associated fibrils in the diagnosis of scrapie in sheep. *Vet. Rec.* **120**, 125-127 (1987).

20

15. Wells, G.A.H., Scott, A.C., Wilesmith, J.W., Simmons, M.M. & Matthews, D. Correlation between the results of a histopathological examination and the detection of abnormal brain fibrils in the diagnosis of bovine spongiform encephalopathy. *Research. in. Veterinary. Science.* **56**, 346-351 (1994).

25

16. Farquhar, C.F., Somerville, R.A. & Ritchie, L.A. Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J. Virol. Meth.* 24, 215-221 (1989).

5 17. Mohri, S., *et al.* Immunodetection of a disease specific PrP fraction in scrapie-affected sheep and BSE-affected cattle. *Vet. Rec.* 131, 537-539 (1992).

10 18. Groschup, M.H., Langeveld, J. & Pfaff, E. The major species-specific epitope in prion proteins of ruminants. *Arch. Virol.* 136, 423-431 (1994).

15 19. Farquhar, C.F., *et al.* A review of the detection of PrPSc. in *Transmissible spongiform encephalopathies* (eds. Bradley, R. & Marchant, B.) 301-313 (Commission of the European Communities, Brussels, Belgium, 1993).

20 20. Vankeulen, L.J.M., *et al.* Immunohistochemical detection and localization of prion protein in brain-tissue of sheep with natural scrapie. *Veterinary Pathology* 32, 299-308 (1995).

25 21. Beekes, M., *et al.* Western-blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method. *J. Gen. Virol.* 76, No.Pt10, 2567-2576 (1995).

22. Vankeulen, L.J.M., *et al.* Immunohistochemical Detection Of Prion Protein In Lymphoid- Tissues Of Sheep With Natural Scrapie. *Journal Of Clinical Microbiology* 34, 1228- 1231 (1996).

23. Wells, G.A.H., *et al.* Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Veterinary Record* **142**, 103-106 (1998).

5

24. Fraser, H., Bruce, M.E., Chree, A., McConnell, I. & Wells, G.A. Transmission of bovine spongiform encephalopathy and scrapie to mice. *J. Gen. Virol.* **73**, 1891-1897 (1992).

10 25. Dawson, M., Wells, G.A.H. & Parker, B.N.J. Preliminary evidence of the experimental transmissibility of bovine spongiform encephalopathy to cattle. *Vet. Rec.* **126**, 112-113 (1990).

15 26. Anderson, R.M., *et al.* Transmission dynamics and epidemiology of bovine spongiform encephalopathy (BSE) in cattle in Great Britain : Past, present and future. *Nature* **382**, 779-788 (1996).

20 27. Taylor, D.M., Ferguson, C.E., Bostock, C.J. & Dawson, M. Absence of disease in mice receiving milk from cows with bovine spongiform encephalopathy. *Vet. Rec.* **136**, 592 (1995).

25 28. Fraser, H. & Foster, J.D. Transmission to mice, sheep and goats and bioassay of bovine tissues. in *Transmissible Spongiform Encephalopathies* (eds. Bradley, R. & Marchant, B.) 145-159 (Commission of the European Communities, Brussels, Belgium, 1993).

29. Bolton, D.C., *et al.* Molecular location of a species-specific epitope on the hamster scrapie agent protein. *J. Virol.* **65**, 3667-3675 (1991).

30. Foster, J.D., Wilson, M. & Hunter, N. Immunolocalization Of the Prion Protein (Prp) In the Brains Of Sheep With Scrapie. *Veterinary Record* 139, 512-515 (1996).

5

31. Hope, J (1998). Transmissible spongiform encephalopathies in man and animals. In Emerging Infections, R. Krause, ed. (New York: Academic Press), pp. 447-461.

CLAIMS

1. An immunometric method for the determination of prion protein (PrP) in a body tissue or body fluid sample from a bovine animal, said method comprising the steps
5 of

- subjecting the PrP to a treatment in order to facilitate its extraction and binding to antibodies, and
- subjecting the PrP from the foregoing step to
 - a) a capture antibody bound or capable of binding to a solid phase, and
 - 10 b) a detecting antibody, and
- quantifying the signal from the detecting antibody bound to the solid phase.

2. The method according to claim 1 wherein the treatment is limited proteolysis.

15 3. The method according to claim 2 wherein the PrP is treated with a proteolytic enzyme such as Proteinase K.

4. The method according to claim 1, 2 or 3 wherein the capture antibody and the detecting antibody are monoclonal antibodies.

20

5. The method according to claim 4 wherein the capture antibody is FH11 and the detecting antibody is 3F4.

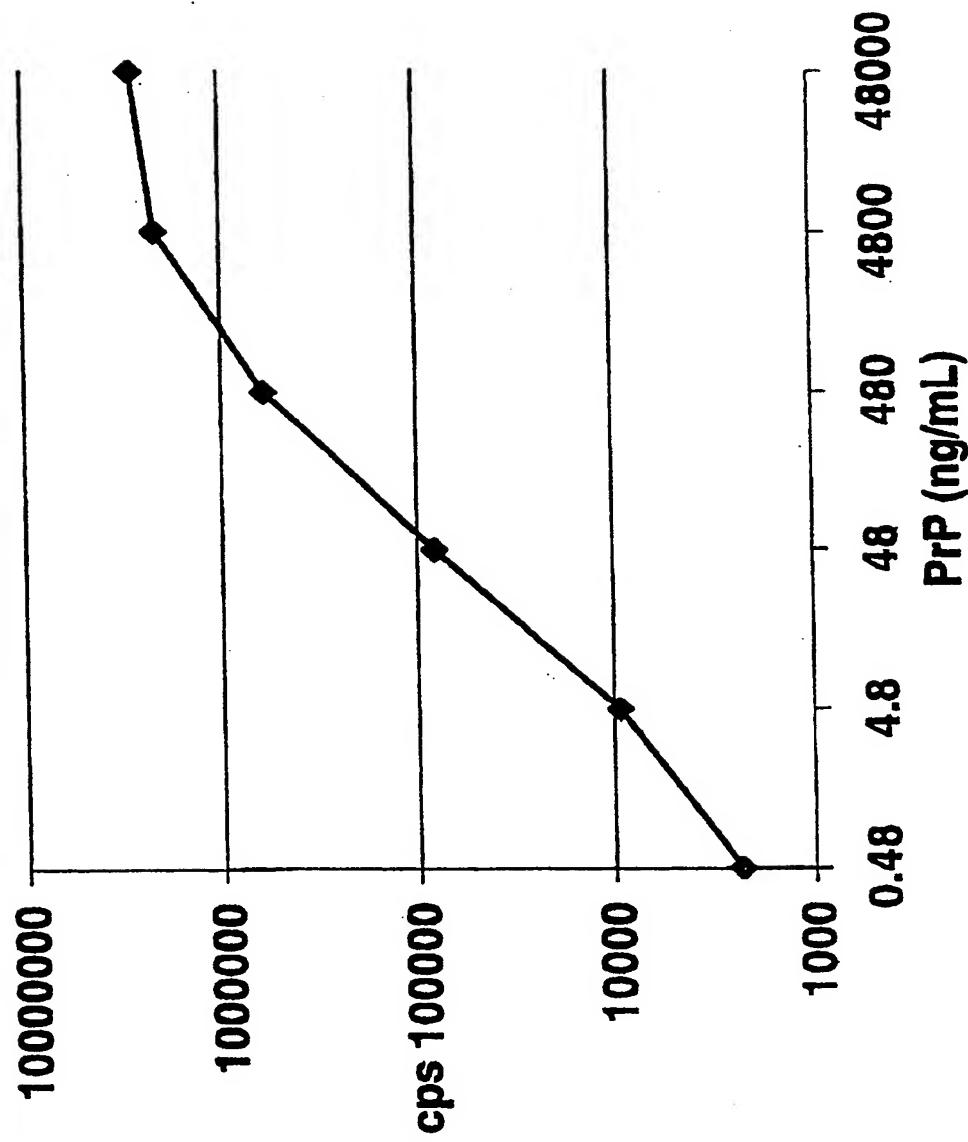
25 6. The method according to any of the foregoing claims wherein the detecting antibody is labelled with a lanthanide chelate.

7. The method according to any of the foregoing claims wherein the sample is brain tissue, spinal cord, lymphoid tissue, spleen, tonsil, whole blood or a blood fraction.

8. A method for diagnosing a bovine spongiform encephalopathy (BSE) in a bovine animal, said method comprising the determination of prion protein (PrP) in a body tissue or body fluid sample from said bovine animal, by a method according to any of the claims 1- 7, wherein the presence of PrP is used as indication of BSE in said
5 bovine animal.

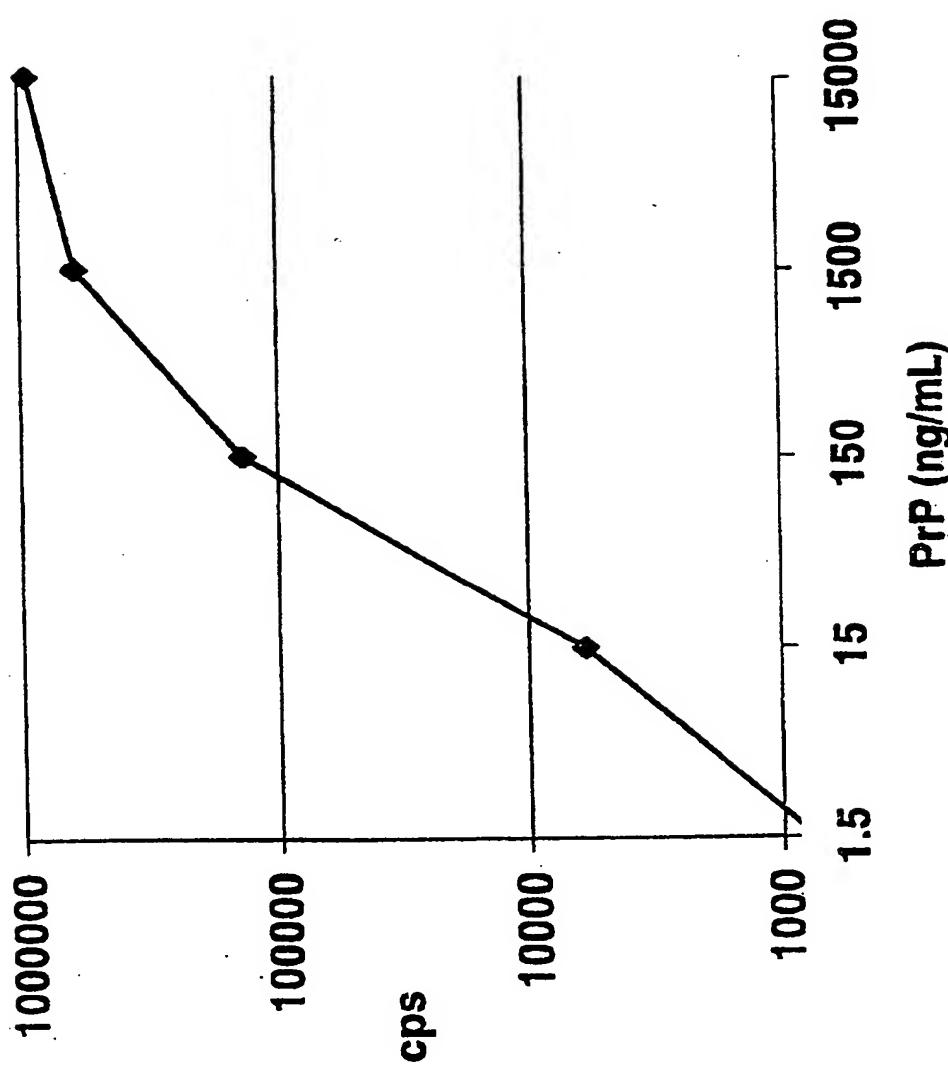
9. A method for the *post-mortem* control of bovine bodies after slaughter in order to identify bodies showing BSE infection, said method comprising the determination of prion protein (PrP) in a body tissue or body fluid sample from said bovine body,
10 by a method according to any of the claims 1- 7, wherein the presence of PrP is used as indication of BSE in said bovine body.

Figure 1: rec. Hamster PrP
Calibration Curve



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Figure 2: rec. Bovine PrP
Calibration Curve



3/5

Figure 3: DELFIA of Hamster PrP Secondary Extract: 3F4 detection

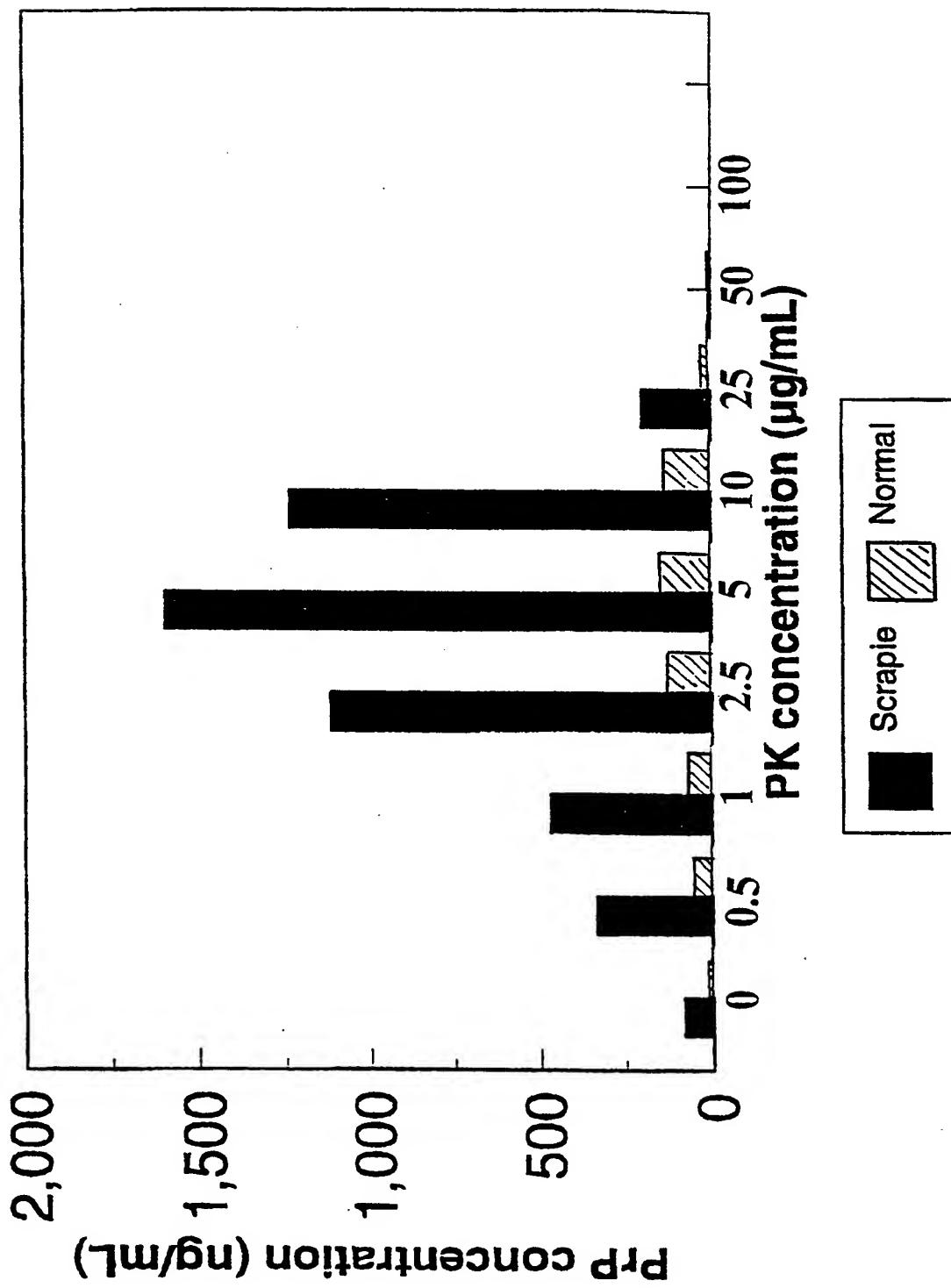
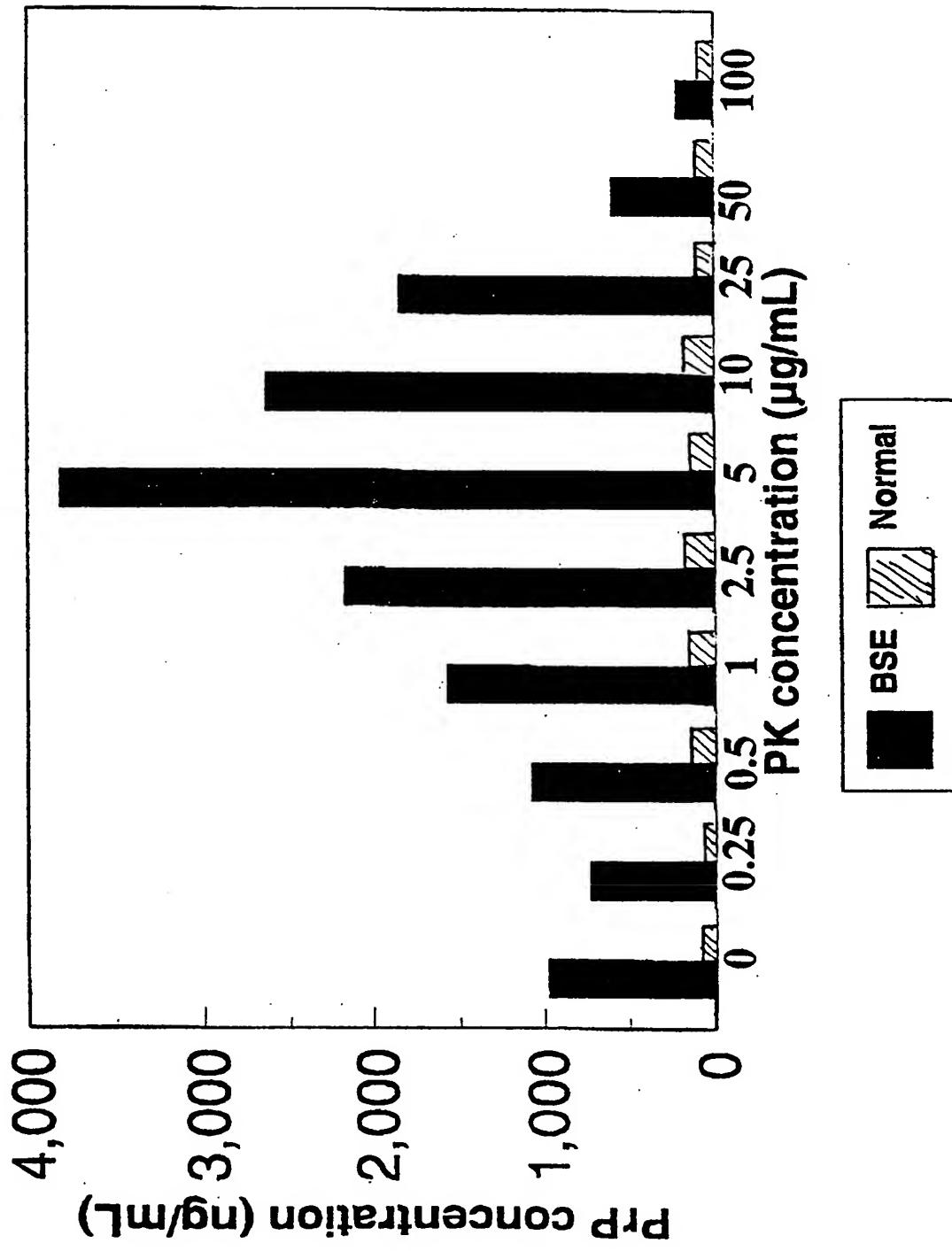
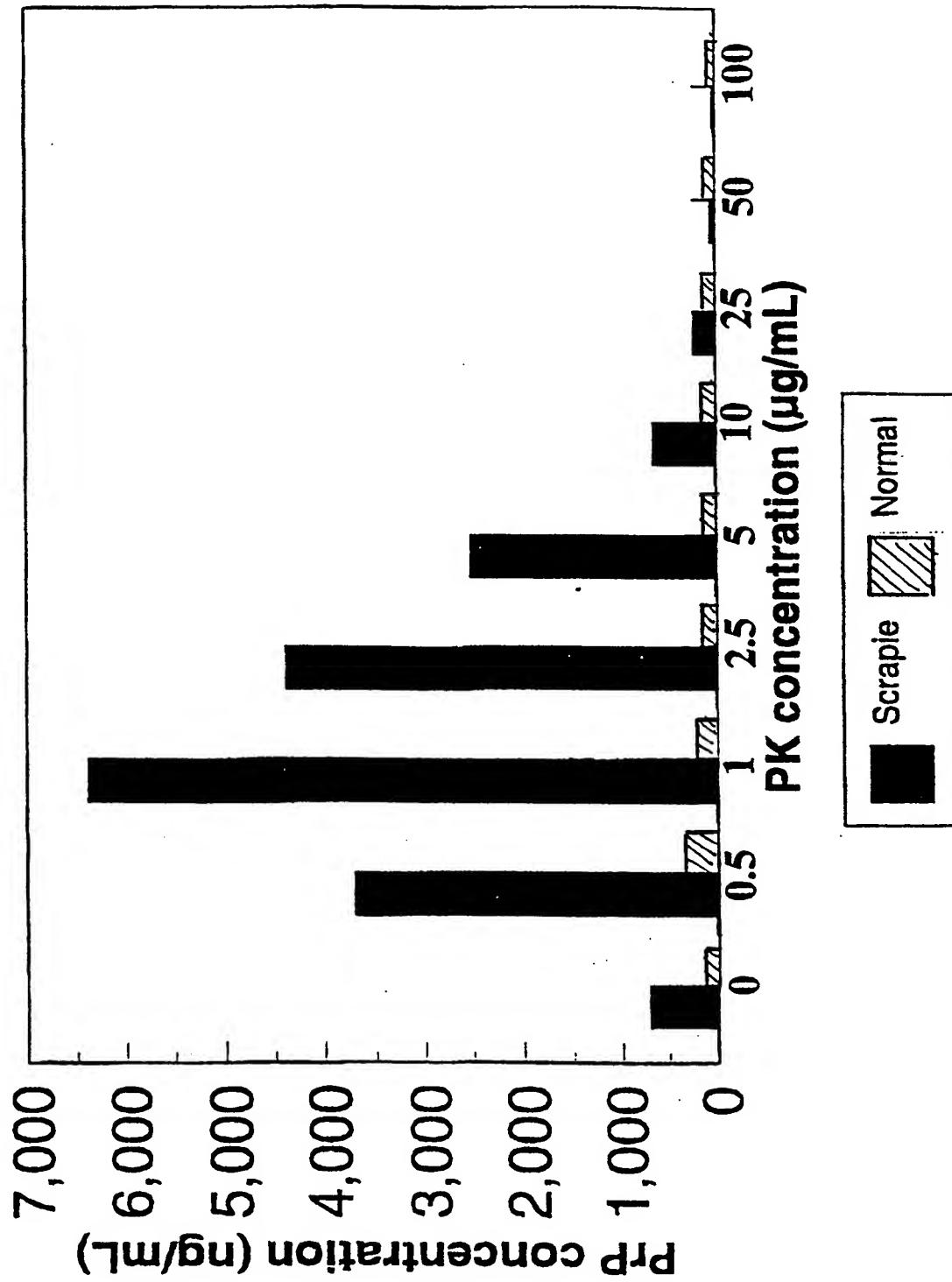


Figure 4: DELFIA of Bovine PrP Secondary Extract: 3F4 Detection



5/5

**Figure 5: DELFIA of Ovine PrP
Secondary Extract: 3F4 detection**



SEQUENCE LISTING

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<120> An Immunoassay for the Determination of Transmissible Spongiform Encephalopathies in Bovine

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00897

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/68, G01N 33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9323432 A1 (NEW YORK UNIVERSITY ET AL), 25 November 1993 (25.11.93), page 4, line 10 - line 36; page 6, line 1 - line 5; page 11, line 37 - page 12, line 6, page 12, line 36 - page, 13, line 14; page 20, line 1 - line 9; abstract	1-4,6-9
Y	--	5
X	EP 0861900 A1 (ERZIEHUNGSDIREKTION OF THE CANTON ZURICH), 2 Sept 1998 (02.09.98), page 9, line 39 - page 10, line 25, abstract	1-4,7-9
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 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents:
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Date of the actual completion of the international search
23 March 2000Date of mailing of the international search report
28.03.2000Name and mailing address of the ISA/
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 99/00897

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9710505 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 20 March 1997 (20.03.97), page 42, line 1 - line 27, abstract --	1-4,7-9
X	GB 2319607 A (ELECTROPHORETICS INTERNATIONAL PLC), 27 May 1998 (27.05.98), page 2, line 10 - page 5, line 20 --	1-4,7-9
Y	Dialog Information Service, File 155, Medline, Dialog accession no. 08961308, Medline accession no. 97111955, Foster JD et al: "Immunolocalisation of the prion protein (PrP) in the brains of the sheep with scrapie", Vet Rec (ENGLAND) Nov 23 1996, 139 (21) p 512-5, ISSN 0042-4900 see abstract --	5
A	US 4806627 A (H.M. WISNIEWSKI ET AL), 21 February 1989 (21.02.89), column 3, line 1 - line 6 -- -----	5

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.	
PCT/FI 99/00897	

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO	9323432	A1	25/11/93	AU 4376093 A	13/12/93
EP	0861900	A1	02/09/98	AU 6498698 A	09/09/98
				WO 9837210 A	27/08/98
WO	9710505	A1	20/03/97	AU 707484 B	08/07/99
				AU 7073596 A	01/04/97
				CA 2231409 A	20/03/97
				EP 0852011 A	08/07/98
				NZ 318689 A	25/02/99
				US 5846533 A	08/12/98
GB	2319607	A	27/05/98	AU 5059798 A	22/06/98
				EP 0937260 A	25/08/99
				GB 9624411 D	00/00/00
				WO 9823962 A	04/06/98
US	4806627	A	21/02/89	NONE	